Exploring the limit of metazoan thermal tolerance via comparative proteomics: thermally induced changes in protein abundance by two hydrothermal vent polychaetes

Exploring the limit of metazoan thermal tolerance via comparative proteomics: Thermally induced expression shifts in two hydrothermal vent polychaetes

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Abstract

Eukaryotic thermotolerance is challenged at deep-sea hydrothermal vents, where temperatures can reach 300 °C. *Paralvinella sulfincola*, an extremely thermotolerant vent polychaete, and *P. palmiformis*, a congener with a more modest thermal tolerance, both flourish at vents along the Juan de Fuca Ridge, Washington, USA. We conducted a series of shipboard, high-pressure, thermotolerance experiments on both species to examine the physiological adaptations that confer pronounced thermotolerance. Quantitative proteomics, a deeply sequenced EST library, and glutathione (an antioxidant) assays revealed that *P. sulfincola* exhibited an upregulation in the synthesis and recycling of GSH with increasing temperature, downregulated NADH and succinate dehydrogenases (key enzymes in oxidative phosphorylation) with increasing temperature, but maintained elevated levels of heat shock proteins (HSPs) across treatments. In contrast, *P. palmiformis* exhibited more typical responses to increasing temperatures, e.g. increasing HSPs at higher temperatures. These data, among the first to quantify global protein and antioxidant responses to temperature in an extremely thermotolerant eukaryote, suggest that *P. sulfincola's* pronounced thermal tolerance is largely due to its capacity to mitigate oxidative stress via increased synthesis of antioxidants and decreased flux through the mitochondrial electron transport chain. Ultimately oxidative stress may be the key factor in limiting all metazoan thermotolerance.

Keywords: Proteomics, Hydrothermal vents, Thermotolerance, Oxidative stress, *Paralvinella*

Introduction

Physiological adaptations to thermal stress are ubiquitous among all organisms. While prokaryotes have a known upper thermal limit of at least 122 °C (1), metazoans have a much lower thermal tolerance, with 45 to 47 °C as the currently accepted upper limit of homeostasis (2), though unicellular eukaryotic fungi are known to grow at 60-62 °C (3). Mitochondrial dysfunction (4), membrane instability, structural disintegration (2), limitations in gas transport and mitochondrial dysfunction have all been implicated as possible modes of physiological failure in eukaryotes (5-7).

There have been numerous studies to date on metazoan thermotolerance (for reviews see: (8, 9)). The few that have focused on highly thermotolerant animals such as desert ants and hot spring ostracods have largely examined their response to acute thermal exposure (10, 11). Recently, some studies have employed proteomics to examine responses to thermal stress in mesotolerant animals (6, 12); however, there remains a limited amount of biomolecular data for extremely thermotolerant metazoans (13). Specifically, it remains to be determined how highly thermotolerant organisms respond to chronic thermal exposure, and which physiological or biochemical adaptations enable them to ameliorate physiological perturbations that arise at higher temperatures.

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Deep-sea hydrothermal vents are ideal habitats to address such questions, as these environments are home to some of the most thermotolerant animals known. This includes the polychaetes *Alvinella pompejana* and *Paralvinella sulfincola*. To date, numerous studies have investigated the thermal tolerance of *A. pompejana*, beginning with the observation that *A. pompejana* lives upon 81° C substratum (14). Subsequent to that, and in contrast to the in situ

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observations, *in vitro* research on *A.pompejana* has suggested key enzymes and structural components are not stable after chronic exposure to elevated temperatures (15). A recent study of *A. pompejana* protein expression via 2D gel electrophoresis compared physiological responses to different oxygen concentrations (13), though its response to chronic exposure remains unconstrained. Notably, *A. pompejana* are not easily amenable to *in vivo* experimentation (16), making it difficult to address chronic thermal tolerance in this species.

Paralvinella sulfincola is another highly thermotolerant polychaete that thrives on hydrothermal sulfides in the Northwest Pacific. *P. sulfincola* are found on sulfide where temperatures reach 88.5°C (17), and *in vivo* laboratory studies of *P. sulfincola* have experimentally demonstrated the broadest known range of chronic thermal tolerance in metazoans (5-48 °C) (18, 19) and (electronic supplementary material –ESM– Figure S1). *P. palmiformis* - a closely related congener - is also found in these environs but exhibits markedly different thermal tolerances (ESM Figure S1). Both are amenable to *in vivo* recovery and laboratory experimentation, which affords the unique opportunity to elucidate the biochemical responses of meso- and thermotolerant metazoans in a comparative phylogenetic context.

To better understand the biochemical mechanisms that underlie extreme thermal tolerance, we present data from a series of *in vivo* high-pressure laboratory experiments in which we examined quantitative changes in protein expression of live *P. sulfincola* and *P. palmiformis* over their thermal range, including temperatures near each species' ultimate incipient lethal temperature (UILT, defined here as the temperature beyond which 50% of the population cannot survive indefinitely (20, 21)). These data reveal statistically significant differences in protein abundance and upregulation between these two congeners, related to mitigating antioxidant stress across their thermal ranges and at their respective UILTs. These data further reveal key

differences in antioxidant concentrations in each species. The results of this study provide the first direct empirical evidence that oxidative stress may be the primary stressor at *P. sulfincola's* upper temperature limit, and illustrates the means by which *P. sulfincola* mitigates this stress.

Results and Discussion

The data herein comprise A) the first extensive assessment of *P. sulfincola* and *P. palmiformis* chronic thermal tolerance; B) a thorough interrogation of their proteomes at chronic, environmentally relevant temperatures using quantitative, high-throughput mass spectrometric sequencing, and C) a comparison of antioxidant production between the two congeners under thermal stress. A normalized expressed sequence tag (EST) library served as the database for the proteomic analyses (due to the qualitative nature of these EST data, as well as the explicit focus of this study on quantitative differences in expression, all data shown here are from the proteomic analyses unless otherwise noted). Together these data reveal that *P. sulfincola* and *P. palmiformis* exhibit overlap in their thermal tolerance ranges, possess markedly different tolerances at their upper and lower bounds, and employ different physiological "strategies" to mitigate thermal stress. Near its UILT, *P. sulfincola* maintains elevated expression of heat shock proteins (HSPs) across its thermal range, rapidly resynthesizes reduced glutathione, and likely decreases oxidative phosphorylation to mitigate the impact of oxygen radicals. In contrast, *P. palmiformis* exhibited responses to chronic thermal exposure that are more similar to those observed in previous studies of mesotolerant organisms, including increased representation of heat shock proteins and other systems solely upon exposure to their highest chronic thermal regimes.

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While we cannot infer metabolic flux from these data (discussed below), the observed systemic differences elucidate those physiological and biochemical processes most responsive to thermal stress. The data suggest that the upper temperature limits of metazoan life may indeed be governed by the ability of the organism to mitigate oxidative stress by managing antioxidant production and vital energy yielding metabolic pathways. The sections below discuss in greater detail the observed differences in protein and antioxidant expression between these two sister taxa.

Differences in Expression of Molecular Chaperones

Molecular chaperones such as heat shock proteins (HSPs) mitigate thermal stress by minimizing protein dysfunction through catalyzing nascent protein folding in the endoplasmic reticulum (ER), reforming misfolded proteins, as well as other functions (22, 23). While many chaperones are constitutively expressed, a large number of chaperones are up-regulated during periods of cellular stress, so-called inducible forms (24). A total of 27 chaperones and co-chaperones were examined in our analysis, representing members of all detected heat shock proteins. Key protein families are discussed in the paragraphs below, and their representation and Bayesian significance are presented in Table 1a, ESM S1 and S2). Briefly, we observed that *P. sulfincola* exhibited elevated levels of all major chaperones, even those previously categorized as inducible, over all treatments (Figure 1), while *P. palmiformis* exhibited higher chaperone production primarily near the UILT.

Heat shock protein 70 (HSP70)

The 70 KDa heat shock proteins (HSP70 family) are the first characterized and best understood chaperones, and are highly conserved across domains of life (23). Multiple isoforms in the family are constitutive, while others are induced by heat stress (24, 25). In *P. sulfincola*, GRP75 proteins, a member of the HSP70 family, exhibited the highest abundance of all molecular chaperones across all *P. sulfincola* treatments. GRP75 is homologous to the human HSPA9, a constitutive mitochondrial HSP (26). In contrast, GRP75 expression in *P. palmiformis* was comparable across many treatments (though there was a moderate increase in expression of GRP75 at 38°C relative to the cooler thermal regimes). A number of co-chaperones that interact with HSP70 family were also observed in all proteomes, and though their expression varied the overall trend for both species was a slight increase in the high treatments.

When *P. sulfincola* peptide sequences were compared against the broader NCBI non-redundant protein database (ESM table S3), peptides homologous to inducible HSP70s were detected, and their relative proportion to total protein remains consistent with constitutive HSP70 proteins. Moreover, in our *P. sulfincola* EST library, three additional HSP70 sequences with human homologs were recovered, though due to their absence in our proteome data are not considered in the quantitative analyses. Together these data underscore the importance of HSP70 proteins in thermal tolerance of both species, and the continued elevated expression in *P. sulfincola* suggest that HSP70 proteins may be kept abundant to cope with the rapid changes in temperature typically encountered by this species, which includes maintaining physiological function near the organism's UILT.

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HSP90

Although less well characterized than the HSP70 family, HSP90s are known as flexible dimer ATPases that bind to a variety of cellular proteins including steroid hormone receptors, transcription factors, and protein kinases (27, 28). The HSP90 protein GRP94 (a luminal protein associated with the endoplasmic reticulum (29)) was detected in the *P. sulfincola* proteome, exhibiting constitutive expression across all treatments (Probability of differential expression - Pr(DE) 0.11). GRP94 was also observed in the *P. palmiformis* proteome, and its abundance likely increased with temperature (12°C → 38°C - log 1.55, Pr(DE) 0.66). Co-chaperones such as HOP, FKBP52 and others known to play a regulatory role with cytosolic HSP90s were observed in both *P. sulfincola* and *palmiformis* proteomes. FKBP52 exhibited a highly significant increase with temperature in both worms (Pr(DE) 1.00). HOP, which modulates HSP70/90 interactions, was also upregulated with temperature in both *P. sulfincola* and *P. palmiformis* at their highest treatments (*P.s.* 10°C → 45°C - log 0.98, Pr(DE) 0.82; *P.p.* 12°C → 38°C - log 0.98, Pr(DE) 0.51). The HSP90 activator AHA1 was substantially upregulated at 45°C in *P. sulfincola* (10°C → 45°C - log 3.58, Pr(DE) 1.00) but not in *P. palmiformis*. Notably, the HSP90 inhibitor CDC37 remained constant in *P. sulfincola* and significantly decreased in *P. palmiformis* in higher thermal regimes. The patterns observed here suggest that HSP90 is constitutively expressed in *P. sulfincola*, but activity is regulated in both species through the regulation of activators and inhibitors. These observations are also consistent with the aforementioned hypothesis that *P. sulfincola* maintains a biochemical poise to cope with acute temperature fluctuations.

HSP 60 and HSP27

HSP60 is a *mitochondrial* molecular chaperone known to confer thermal tolerance in eukaryotes (30). Our analysis revealed that HSP60 was the most consistently expressed heat shock protein, with high abundance across all treatments in both species. This trend was mirrored in the HSP60 co-chaperone, HSP10, which assists HSP60 in protein folding during periods of stress (31). These findings suggest that both species maintain pools of HSP60 and HSP10 to mitigate damage to mitochondrial proteins.

The small 27kDa heat shock protein (sHSP), found throughout cellular compartments and the cytosol, responds to both thermal and oxidative stress by binding to damaged or misfolded proteins and forming reservoirs for other chaperones to correctly refold or initiate proteolytic degradation (32). It is also known to upregulate key enzymes in the glutathione pathway (32, 33). HSP27 was abundant across all treatments in *P. sulfincola.* However, HSP27 increased only at the highest temperature in *P. palmiformis* (12°C → 38°C - log 2.09, Pr(DE) 1.00). We posit that the differences observed between expression levels of HSP27 relate to oxidative stress response and the glutathione pathway (discussed in detail below).

Foldases

Foldases are enzymes that catalyze rate-limiting steps in protein folding, many of which play a key role in the cellular "unfolded protein response" (a stress response to an accumulation of unfolded and misfolded proteins in the endoplasmic reticulum, which aims to restore normal function by halting protein translation and signaling the production of molecular chaperones involved in protein folding; (34)). Foldases important to the UPR were detected in both species. Of note, the foldase PDIA1, a protein-thiol oxidoreductase that acts as both a chaperone and a foldase (34, 35), was abundant (constitutive) across all treatment in *P. sulfincola* (Pr(DE) 0.001).

189 In *P. palmiformis*, PDIA1 abundance increased as a function of temperature (Pr(DE) 0.914),
190 reinforcing the pattern of differential response observed between these two organisms in relation
191 to thermal stress.

192 While the data on chaperones demonstrate that *P. sulfincola* maintains elevated
193 expression of chaperones across all thermal regimes, we posit that the representation and
194 abundance of chaperones does not itself explain the observed thermotolerance. Indeed, if HSP
195 abundance alone was the key factor in conferring extreme thermotolerance, then *P. palmiformis*
196 would likely have a greater thermal tolerance similar to *P. sulfincola* (with a UILT above 38°C)
197 because the representation of chaperones between these two closely related species was
198 (proportionally) equivalent at their respective highest thermal treatments. We therefore further
199 posit that elevated HSP abundances in *P. sulfincola* are more likely a reflection of its ecological
200 niche *in situ*, enabling it to survive acute, rapid shifts in temperatures caused by its proximity to
201 hot vent fluid, but does not alone explain their chronic thermal tolerance.

202 *Response to Oxidative Stress*

203 The largest shifts in protein abundance observed in both species are related to the
204 mitigation of oxidative stress. In mitochondria, the reactive oxygen species superoxide ($O_2^{\bullet-}$) is
205 generated in complexes I/III during respiration, and other ROS such as in the hydroxyl radical
206 ($HO^{\bullet}$), and uncharged hydrogen peroxide (H_2O_2), are produced in the outer and inner membranes
207 (for review see (36, 37)). Under normative conditions, mitochondria consume more than 90% of
208 all cellular O_2, while also producing the majority of ROS (38). However, studies have shown that
209 elevated temperatures can also increase oxidative stress in mesotolerant eukaryotes (5, 39, 40), as

elevated temperatures increase the metabolic demand of tissues, induce a state of functional tissue hypoxia, and increase mitochondrial respiration rates (41).

Superoxide dismutase (SOD, EC 1.15.1.1) is a ubiquitous enzyme that is responsible for catalyzing the reduction of $O_2^{\bullet-}$ to H_2O_2. There are two forms of this metalloprotein; Cu/Zn SOD (isotig03775) are primarily found in the cytosol, whereas Mn SOD (isotig06674) are located the mitochondria (42). *P. sulfincola* showed no differences in the abundance of either SOD across all treatments, but *P. palmiformis* exhibited significant increases in both Mn SOD (Pr(DE) 0.997) and Cu/Zn SOD (Pr(DE) 0.999) (ESM tables S1, S2).

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In *P. sulfincola,* however, the production of glutathione appears to play a prominent role in mitigating ROS. Glutathione (L-γ-glutamyl-L-cysteinylglycine, or GSH) is a tripeptide thiol that is the primary nonprotein antioxidant in metazoans. Found in up to mM concentrations in mammals, GSH mitigates oxidative stress by chemically reducing hydrogen peroxide and other toxic compounds (36, 43). The enzyme glutathione peroxidase (GPx, 1.11.1.9) catalyzes this reduction, yielding glutathione disulfide (GSSG). GSSG is reverted back to GSH by glutathione reductase (GSR, EC 1.8.1.7). Regulation of GSH metabolism and resynthesis serves as an indicator of cellular oxidative stress levels (43). As cysteine is the required peptide for *de novo* GSH synthesis, and the rare amino acid selenocysteine is required for the synthesis of glutathione peroxidase, increases in cysteine and in particular selenocysteine are good indicators for increases in GSH cycling (Table 1b, ESM Tables S1 and S2).

Figure 2 depicts key steps and significant changes over temperature in the synthesis of glutathione, the redox cycle of GSH and GSSG, and the catalyzing enzymes glutathione peroxidase (GPx) and glutathione reductase (GSR) in *P. sulfincola* and *P. palmiformis*. Notably,

cystathionine beta-synthase (CBS, EC 4.2.1.22), central to both cysteine and selenocysteine synthesis, exhibited the single largest fold increase with temperature of all proteins assayed in *P. sulfincola* and nearly so for *P. palmiformis* (*P.s.* 10°C → 45°C - log 5.74, Pr(DE) 1.00; *P.p.* 12°C → 38°C - log 5.55, Pr(DE) 1.00). Two ATP-dependent, rate-governing steps within the glutathione pathway were detected in our *P. sulfincola* and *P. palmiformis* proteomes: selenide water dikinase (selD, EC 2.7.9.3), and gamma-glutamylcysteine synthetase (GCS, EC 6.3.2.2) (Figure 2). SelD, essential for *de novo* synthesis of selenoproteins, increased in *P. sulfincola* at both 30°C and 45°C, while no differences in abundance were observed in *P. palmiformis*. GCS, the rate-limiting step in the production of GSH and subject to feedback inhibition (43), showed a steady increase in abundance with temperature in *P. sulfincola*. In *P. palmiformis*, however, GCS was not detected until 38°C treatment, producing a significant correlation with temperature (12°C → 38°C - log 3.81). These data clearly suggest that GSH is being synthesized at higher rates in response to increasing thermal stress in both species, though far more pronounced in *P. sulfincola*.

Glutathione peroxidase 3 (GPx-3, cytosolic) showed significant increases in abundance at both the medium and high temperature treatments in *P. sulfincola*, as well as at the highest temperature treatment in *P. palmiformis*. Notably, *P. sulfincola* significantly increases its GSR protein abundance while *P. palmiformis* significantly decreases it at higher temperatures. These data suggest that *P. sulfincola* is continuously recycling GSH in the mitochondria. We further suggest that the differences may be indicative of mitochondrial dysfunction and uncoupling in *P. palmiformis*, possibly due to lipid peroxidation from increasing ROS activity, as has previously been observed in cold-water marine mollusks exposed to heat stress and functional hypoxia (5, 38).

To further investigate the effect of thermal and oxidative stress on the pool of GSH, total GSH (GSHt) levels and GSH/GSSG ratios (the ratio of the reduced and oxidized forms) were measured for medium and high temperature treatments in both species (ESM Figure S2). GSHt concentrations in *P. sulfincola* were about half those observed in *P. palmiformis.* There were no measurable differenced in the GSH/GSSG ratio among *P. sulfincola* worms across all thermal treatments. However, in higher thermal treatments, *P. palmiformis* exhibited a 2-fold decrease in the pool of GSHt. Furthermore, the GSH/GSSG ratio in *P. palmiformis* exhibited more than a 3-fold drop at higher thermal treatments, indicating that *P. palmiformis* were not able to effectively recycle glutathione at 38°C. These trends suggest that *P. sulfincola* is well poised to sustain GSH resynthesis near its UILT, allowing it to maintain functionality even under periods of high oxidative stress (the limited sample size prohibited statistical analyses of these observations).

Oxidative Stress and Oxidative Phosphorylation

In eukaryotes, oxidative phosphorylation within the electron transport chain is responsible for the majority of ATP production and ROS formation. As mentioned, research has indicated that elevated temperature can lead to local tissue hypoxia (41). Here, *P. sulfincola* exhibited a significant reduction in abundance of NADH dehydrogenase (10°C → 45°C - log -2.01) and succinate dehydrogenase (10°C → 45°C - log -1.00), both of which are involved in the mitochondrial oxidative phosphorylation (Tables S1 and S2). Indeed, a large portion of ROS is generated by NADH dehydrogenase (complex I). Succinate dehydrogenase (complex II) may not contribute directly to ROS formation, but it funnels electrons to complex III, which does produce ROS. In *P. palmiformis,* the decrease in NADH dehydrogenase was less pronounced (12°C → 38°C - log -0.48), and there was an increase in abundance of succinate dehydrogenase with temperature (12°C → 38°C - log 1.53). The observed patterns of NADH dehydrogenase

278 contrasts with previous studies of a heat sensitive mussel species *Mytilus galloprovincialis,* ,
279 which increased production of NADH dehydrogenase relative to its less thermotolerant congener
280 *Mytilus trossulus* (6). These data suggest that *P. sulfincola* may be actively repressing ROS
281 formation at high temperatures by lessening endogenous generation *via* the ETC, and depending
282 more heavily on anaerobic respiration at elevated temperatures.

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283 *Global proteome responses, emerging hypotheses and future directions*

284 Quantitative mass spectrometric protein analyses reveal hundreds of differentially
285 expressed proteins per treatment, yet efforts to ally proteomic (or transcriptomic) data to
286 metabolic rate have met with limited success (44, 45). This is likely attributable to the
287 complexity of interactions among enzymes, their subtrates and other factors that regulate flux
288 through a pathway. Gross changes among metabolic pathways, however, provide another –albeit
289 coarser- means of assessing organismal response to thermal stress as it reveals broad trends in
290 the abundance of proteins allied to specific systems. iPath (46) was used to map changes in
291 global protein abundance within 139 KEGG metabolic pathways, and reveal significant
292 (posterior probability <0.05), broad and complex differences in protein expression between
293 species and among treatments (ESM Figure S3a-d, S4). Protein upregulation in *P. palmiformis*
294 between the 21°and 38°C treatments is significantly higher than in other treatments. It is
295 possible that *P. palmiformis* is incapable of maintaining homeostasis at the higher temperature,
296 and is exhibiting metabolic disorder, as evident by the changes in the TCA and pentose
297 phosphate cycle, both known to respond to thermal stress (ESM Figure S5) (6, 12). At their
298 highest temperature treatments, *P. sulfincola* and *P. palmiformis* exhibited opposing patterns of
299 protein expression in the TCA cycle, with *P. sulfincola* decreasing and *P. palmiformis* increasing

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expression of enzymes respectively. In the pentose phosphate pathway, *P. sulfincola* and *P. palmiformis* again exhibited opposing patterns of expression, exhibiting increased and decreased enzymes respectively. Depression of the TCA cycle in *P. sulfincola* may be due to thermal effects on energy metabolism, or may be attributable to the oxygen concentrations in our experiments, which at 130 µM are comparable to ambient bottom water but higher than some diffuse flows (discussed below). Nevertheless, these trends are consistent with a decreased emphasis on aerobic respiration (TCA cycle) and the need for reducing equivalents to maintain sufficient GSH for antioxidant activity (pentose can be converted into glucose 6-phosphate to produce NADPH to recycle oxidized GSH (47)). Further targeted studies may better reveal correlations between flux rates and protein counts, helping our understanding of the effects of thermal stress on metabolic processes. In addition, future studies should also consider the impact of post-translational protein modifications to physiological functions at elevated temperatures.

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Conclusions

These data lead us to conclude that *P. sulfincola* maintains a pool of both canonical constitutive and inducible heat shock proteins to maintain protein function during rapid and frequent exposure to high temperatures in its highly dynamic environment. Notably, enzymes and pathways associated with the production of antioxidants showed the most pronounced response to thermal exposure in both *P. sulfincola* and *P. palmiformis*. However, only *P. sulfincola* increased the abundance of enzymes responsible for chemically reducing GSSG, thereby maintaining its antioxidative capacity. Increasing the *de novo* synthesis of GSH from the cysteine pathway (and the catalytic enzyme GPx through increases in selenocysteine) at elevated temperatures further underscores this pathway's relevance in oxidative scavenging. Increased

322 production of GSR, necessary for recycling GSSG also demonstrates that *P. sulfincola* maintains
323 a sufficient pool of GSH to mitigate oxidative stress. The concurrent decreases in *P. sulfincola*
324 enzymes associated with oxidative phosphorylation may reduce the rate of oxidative radical
325 formation at high temperature.

326 In contrast, *P. palmiformis* exhibited significant increases in major molecular chaperones
327 with increasing temperature, and increases in other systems including the production of
328 antioxidants. However, *P. palmiformis* did not exhibit a comparable capacity to regenerate GSH
329 at elevated temperatures, or reduce the production of ROS from oxidative phosphorylation.
330 Indeed, *P. palmiformis* responded in a manner similar to other comparatively mesotolerant vent
331 endemics such as *Paralvinella grasslei* (16) and *Rimicaris exoculata* (48).

332 *P. sulfincola* lives on vent edifices, where they might readily encounter regions of
333 elevated temperature and varying oxygen concentration (due to radiative heating, the water
334 around vent sulfides can be warm yet exhibit a composition more similar to the ambient seawater
335 (49)). In addition, *P. sulfincola* are exposed to more sulfidic fluids as well, which might also
336 affect their thermal tolerance due to the impact of sulfide on aerobic respiration (addressing the
337 role of sulfide in thermal tolerance is beyond the scope of this study, and should be revisited in
338 future investigations). Based on the data presented here, we posit that *P. sulfincola's* pronounced
339 thermotolerance is enabled primarily by adaptations to mitigate oxidative stress, which include
340 increasing activity of antioxidant systems and decreasing aerobic metabolism. We further
341 suggest these patterns demonstrate that managing ROS, resulting from increased mitochondrial
342 aerobic respiration at elevated temperatures, is a high priority for thermotolerant organisms.
343 Considering that all metazoans are ultimately dependent on mitochondrial aerobic respiration,
344 ROS may effectively limit them to cooler thermal regimes than thermophilic bacteria and

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345 archaea (the most thermophilic prokaryotes are anaerobes, and exhibit a striking antioxidant
346 response when exposed to modest amounts of oxygen (50)). Although oxidative stress has been
347 implicated in previous studies on mesophilic eukaryotes (2, 5, 6, 41), this is the first study to
348 empirically derive this link between the UILT and ROS production in one of the most
349 thermotolerant metazoans on the planet, suggesting that oxidative stress -not temperature itself-
350 may limit metazoan thermal tolerance.

351 **Materials and Methods**

352 *Animal collection and experimental apparatus*

353 *Paralvinella sulfincola* and *Paralvinella palmiformis* "palm worms" were collected from
354 hydrothermal vents in the Main Endeavour field along the Juan de Fuca Ridge (47°57'N,
355 129°5'W) at a depth of 2,200m during the R/V *Atlantis* expedition 15-34 in July 2008.
356 Organisms were collected by the DSV *Alvin* on dives #4409-4423, using either a multi-chamber
357 suction sampler or an insulated sample recovery box. Upon recovery to 1 atm, worms were
358 transferred to a 4°C cold room and visually sorted based on segment number and gill
359 morphology. Aggregations of mucus and minerals were removed from the animals before
360 transfer into a flow-through high pressure aquaria system.

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361 *High-pressure aquaria system*

362 *P. sulfincola* incubations were conducted in a newly designed high pressure aquaria
363 system (ESM Figure S6). *P. palmiformis* low temperature incubations (12°C) were conducted in
364 a 500 cm^3 titanium flow-through system with 50 mL/min flow rate (51). Dissolved oxygen
365 concentration was measured at the inlet and outlet of each system using a polarigraphic oxygen

366 electrode (limits of detection ca. 1 µM; YSI Inc) to verify that oxygen was always greater than
367 25 µM, which is not limiting based on known hemoglobin oxygen binding affinities of
368 alvinellids (52). Dissolved oxygen concentrations were achieved by equilibrating the seawater
369 with air, at the elevated experimental temperatures and 1 ATM. The net effect was a dissolved
370 oxygen concentration of ~ 130 µM, which is slightly higher but comparable to the ambient
371 bottom water concentration at the Juan de Fuca ridge (~120 µM).

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372 *Experimental design*

373 Though critical thermal maxima (CT_{max}) of both species and thermal preference of *P.*
374 *sulfincola* were previously examined (18, 19), we augmented these data to better establish *P.*
375 *sulfincola and P. palmiformis* chronic thermal tolerance (ESM Figure S1). A total of 85 *P.*
376 *sulfincola* and 108 *P. palmiformis* were utilized in this study. Chronic thermal tolerance was
377 defined as a lack of temperature-induced mortality over 12 hours of sustained exposure. On
378 occasion, <5% of individuals died during treatments, which upon further inspection we attributed
379 to recovery and handling. Based on these data, three temperatures were chosen that span the
380 chronic thermal tolerance range of each species (*P. sulfincola* = 10°C, 30°C, and 45°C; *P.*
381 *palmiformis* = 12°C, 21°C, and 38°C; ESM Figure S1). These temperatures, although not
382 identical in their ΔT, were chosen to represent the organisms' protein profiles across their
383 respective thermal tolerance ranges, including temperatures approaching their UILT. We posit
384 that the resulting data better represents protein abundance and their lower, nominal, and upper
385 temperature regimes. At each treatment, six to nine worms were maintained at constant pressure
386 and temperature for >12 hours for global protein expression analysis. To minimize the effects of
387 collection and handling, worms were first acclimated in each system at room temperature (21°C)

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388 for twelve hours prior to experimentation. At the conclusion of each trial, the chambers were
389 quickly depressurized, and worm health was assessed by looking for signs of embolisms, motor
390 dysfunction or other physiological damage that might have arisen from thermal exposure or other
391 experimental handling. Healthy worms were selected, and their branchiae and body tissues were
392 separated and flash frozen in liquid nitrogen for subsequent protein extraction.

393 *Transcriptome Sequencing and Analysis*

394 A *Paralvinella sulfincola* expressed sequence tag (EST) library was sequenced and built
395 by the Joint Genome Institute (Walnut Creek, CA). Briefly, mRNA was purified from total RNA
396 isolated at two different temperature conditions for two tissue types (body and gill). cDNA from
397 each was generated using an oligodT primer followed by template switching (Clontech,
398 Mountain View, CA) and subsequently normalized using the provided protocol of the Evrogen
399 Normalization kit (Evrogen, Moscow, Russia). The normalized cDNA was used to build a library
400 with the construction protocol provided in the 454 Flx Titanium Roche kit (Roche, Branford,
401 CT) and then sequenced. Four EST libraries consisting of 2,593,853 reads were filtered and
402 screened for quality and contamination to produce a filtered set of 2,382,211 reads. These reads
403 were then assembled using Newbler (v2.3-PreRelease-6/30/2009), which resulted in 80748 raw
404 contigs. Herein, contigs are single exon reads, and isotigs are contigs assembled into potential
405 gene assemblies. After a cutoff minimum length of 350 base pairs, the aforementioned sequences
406 were combined to create 24,702 sequences (24,164 isotigs and 538 contigs). The average length
407 of this library is 1,290 bp/sequence and the GC content average is 0.40 (ESM table S4). The
408 sequences were aligned using BlastX with the Swissprot database. 12,562 of the translated
409 sequences had a known BlastX match and 7,002 unique proteins were identified. Longest ORF

Geoffrey Dilly 12/20/11 12:29 PM
Comment: This refers to Rev 1 Question 13

translations were used as the reference library for all subsequent MS/MS oligopeptide spectra. Sequences are available at the NCBI's sequence read archive (SRA; http://www.ncbi.nlm.nih.gov/) under accession # SRA034879.

Protein extraction

Gill branchiae from three *P. sulfincola* and three *P. palmiformis* per treatment were excised, weighed on an electronic balance (Mettler Toledo, Columbus, OH), and placed into sterilized 0.5 mL glass micropestles (Wheaton, Millville, NJ) containing 24 uL of 20mM Tris pH 7.5 buffer and 6 uL Protease Inhibitor Cocktail (PIC) (Sigma-Aldrich, St. Louis, MO). Tissue was homogenized until complete dissociation then centrifuged at 1000x g for 5 minutes. For protein extraction, 0.5 mg gill branchiae were used in a modified Laemmli protein extraction protocol (53). A Tris/PIC mixture at 1:1v/v and 1:20 2-mercaptoethanol/ Laemmli Buffer were added, and the solution was heated at 95°C for 10 minutes. All extractions were loaded in separate lanes onto 4-20% precast Precise Protein Gels (Pierce Inc) with blank lanes between samples. The gels were bathed in a Tris-HEPES-SDS buffer solution and electrophoresed for 45 minutes at 100V. Band size and run length were assessed by including 10uL of BenchMark Pre-Stained Protein Ladder 10-190 kDa (Invitrogen, Carlsbad, CA). After electrophoresis, gels were rinsed and stained for three hours using the colloidal commassie blue dye Novex (Invitrogen, Carlsbad, CA). Gels were visualized using a digital gel imaging system (Kodak Gel Logic 100, Kodak, Rochester, NY) and sub-sectioned into six fragments according to protein size. Three biological replicates from each treatment were pooled into one sample per fragment; total gel surface area did not exceed $1cm^2$. The pooled gel sub-sections were then washed with 1 mL of 50% acetonitrile and frozen at -20°C prior to analysis.

Protein analyses by tandem mass spectrometry

A total of 36 pooled samples (2 species incubated at 3 temperatures fractionated into 6 equal sections) were reduced, carboxyamidomethylated, and digested with trypsin. Resulting peptides from each sample were analyzed over 3 technical replicates using microcapillary reverse-phase HPLC directly coupled to the nano-electrospray ionization source of a ThermoFisher LTQ-Orbitrap XL (replicate 1) or LTQ-Orbitrap Velos (replicates 2 and 3) hybrid mass spectrometer (μLC/MS/MS). The Orbitrap repetitively surveyed *m*/*z* range from 395-1600, while data-dependent MS/MS spectra on the 20 most abundant ions in each survey scan were acquired in the linear ion trap. MS/MS spectra were acquired with a relative collision energy of 30%, 2.5-Da isolation width, and recurring ions dynamically excluded for 60s. Preliminary evaluation of peptide-spectrum matches (PSMs) was facilitated using the SEQUEST algorithm with a 30 ppm mass tolerance against the *P. sulfincola* EST library and NCBI nr databases. Spectral counting is a method of relative quantitation in which one compares the number of MSMS spectra acquired for a particular protein across multiple LC-MS/MS datasets. Increases and decreases in relative protein abundance are reflected in corresponding increases and decreases in spectral counts for that protein (54, 55). PSMs were accepted with mass error <3.0 ppm and score thresholds to attain an estimated false discovery rate of ~1% using a reverse decoy database strategy and a custom version of the Harvard Proteomics Browser Suite (ThermoFisher Scientific, San Jose, CA). A total of 172,122 peptide spectra were identified with an average of 14.6 amino acids/sequence, with MS/MS spectra populating 1296 referenced proteins (ESM table S5).

Glutathione Measurements

454 Total GSH and GSSG levels were measured using the Glutathione Assay Kit (Cayman
455 Chem, Ann Arbor, MI) as per instructions. Spectrophotometric readings were taken kinetically
456 for 30 minutes using a Spectramax Plus^{384} (Molecular Devices, Sunnyvale, CA). Internal
457 standards were run with total GSH and GSSG experimental treatments, and standard curves were
458 built from the endpoint readings.

459 *Data Analysis and Statistics*

460 BaySeq (56) was used to determine statistically significant relative changes over
461 experimental treatments (ESM tables S1 and S2), following methods described in (57). Peptide
462 spectral counts were modeled using a negative binomial distribution to account for potential
463 overdispersion among treatment replicates. By borrowing information on replicate variance
464 among peptides over the entire dataset (ESM Figure S7), the method employed in baySeq better
465 calibrates replicate variance for individual peptides than can be achieved through standard
466 methods of modeling overdispersed count data. Using a likelihood cutoff of 0.9, Bayesian
467 analysis revealed 428 differentially expressed proteins in *Paralvinella palmiformis* and 214
468 differentially expressed proteins in *Paralvinella sulfincola.* We use the convention of a 0.9
469 likelihood cutoff throughout the analysis as in significance indicator, but it is important to note
470 that Bayesian methodology allows for the comparison of relative likelihoods that we explore
471 within the context of each protein family. Additionally, metabolic enzyme regulation was
472 examined *via* pathway analysis. The R package ShotgunFunctionalizeR (Version: 1.0-3, Date:
473 2009-10-09) was used after assigning Enzyme Commission (EC) numbers to sequences using
474 KEGG assignments and the R package BioIDMapper (Version: 2.1, Date: 2010-01-16). To
475 assess statistical support for metabolic pathway-level expression differences, ortholog data were
476 combined into KEGG pathways using methods described in (57). We assumed a binomial

distribution in this case, and Monte Carlo methods were used to determine the posterior probability of differential expression, point estimates of pathway abundance and 95% credible intervals for these estimates. Methods for iPath described in (46).

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627 **Figure Legends**

628 **Figure 1:** Molecular chaperones

629 Differences in expression between *P. sulfincola* and *P. palmiformis* in log fold-change for six
630 major molecular chaperones across their thermal range. S10 →45 = difference from *P. sulfincola*
631 maintained at 10°C to 45°C; P12 →38 = difference from *P. palmiformis* maintained at 12°C to
632 38°C. Stars (*) indicate that the log change is > 0.90 in our Bayesian analysis, indicating a
633 significant change with temperature. We assumed a binomial likelihood for the data and a Beta
634 (0.5,0.5) prior for each treatment. Monte Carlo sampling from the resulting posterior
635 distributions within each treatment was used to estimate the posterior distributions of log-fold
636 changes between treatments. We report the medians and 95% credible intervals (bars) of the
637 posterior distributions of log-fold change between treatments.

638 **Figure 2:** Representative glutathione pathway in *Paralvinella* with responses to thermal
639 exposure

640 Synthesis pathways of the antioxidant glutathione (GSH) and its catalyzing enzyme Glutathione
641 peroxidase (GPx). Ovals represent enzymes; grey rectangles indicate substrates. Grey ovals
642 represent proteins only observed in the *P. sulfincola* EST database. Color indicates significance
643 and direction of regulation. Asterisks indicate ATP-dependent enzymatic steps. Numbers in
644 diamonds correspond to protein count rows in Table 1b. Note: GPx appears twice – in synthesis
645 in the selenium pathway, and in oxidizing GSH to GSSH. DNMT is found in cysteine pathway
646 only; at present, the specific seleno-methyltransferase for Paralvinellids is unknown. Some
647 reaction cofactors omitted for simplicity. Abbreviations: AHCY, Adenosylhomocysteinase A;
648 CBS, Cystathionine β-synthase; CGL, Cystathionine γ-synthase; DNMT, DNA (cytosine-5-)-

649 methyltransferase; GPx, Glutathione Peroxidase; GS, Glutathione synthetase; GSH, Glutathione;
650 GSSH, glutathione disulfide; GSR, Glutathione reductase; GSTs, Glutathione sulfur transferases;
651 MAT2, Methionine adenosyltransferase; SelD, Selenide water dikinase.

652 **Table 1a, b.** Shifts in *Paralvinella* protein abundance during thermal exposure

653 Key enzymes of *P. sulfincola* and *P. palmiformis* discussed in the text. EST refers to the (i) isotig
654 or (c) contig identifier for each enzyme. Log change refers to the shift in abundance between
655 treatments, i.e. (log Δ *P.p* – 12 → 21 = protein log fold change between *P. palmiformis*
656 treatments 12°C and 21°C). Counts are combined between all three technical replicates and
657 normalized to treatment library sizes. Red boxes indicate a significant (Pr(DE) >0.9) increase in
658 protein abundance; Blue boxes indicate a significant (Pr(DE) >0.9) decrease in protein
659 abundance. **Table 1a** lists chaperones; **Table 1b** lists glutathione pathways.